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A membrane-bound protein kinase from mouse liver stimulated by iron

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At μM levels, iron stimulates strongly the in vitro phosphorylation of a membrane protein from mouse liver. This phosphorylation also occurs in the absence of other added divalent cations but to a lower degree. In SDS gels the phosphorylated protein has app. M_r 250000 in the absence of mercaptoethanol and M_r 130000 in its presence. It is phosphorylated on threonine residues.

Membrane, of mouse liver *Protein kinase* *Iron* *Threonine phosphorylation*

1. INTRODUCTION

Iron, as a cofactor of many enzymes, is an element essential for life. It is necessary in cell culture medium for practically all cell types. It is normally in the form of transferrin either in serum or in culture medium without serum. At least in some cases, iron may replace transferrin in artificial culture medium [1–3]. At mM levels, Fe^{2+} can substitute for Mg^{2+} as cofactor for the activation of nuclear casein kinases I and II [4]. Phosphorylation systems at the membrane level play an important role in the transmission of extracellular signals. Thus, we have investigated if Fe^{2+} can also activate membrane protein kinases and have found that in mouse liver membranes, Fe^{2+} at 100 μM activates the in vitro phosphorylation of a single polypeptide chain of app. M_r 130000.

2. MATERIALS AND METHODS

2.1. *Membrane isolation and protein determination*

Blood sinusoidal membrane subfractions were prepared from the liver of normal Swiss male adult mice as in [5], except that 1 mM PMSF and 5 mM iodoacetamide were added to all solutions. A final 27–34% (w/v) sucrose continuous gradient was included in the procedure to obtain sinusoidal membrane-enriched fractions free from smooth microsomes and Golgi vesicles. Sucrose solutions used in the latter gradient were buffered with 5 mM Tris–HCl (pH 7.5) and they contained 1 mM PMSF and 5 mM iodoacetamide. After centrifugation for 2 h at 300000 × g in a Beckman SW50.1 rotor, sinusoidal plasma membranes were collected between 29–34% (w/v) [6]. They were washed with 150 mM NaCl, 50 mM Tris–HCl (pH 7.5) and stored in aliquots (500 μg protein) at −80°C). Protein content was determinated following [7] with bovine serum albumin as standard.

2.2. *Endogenous phosphorylation of membrane proteins*

The assay was performed in 35 μl of reaction mixture containing the following: membranes, 30 μg protein; Tris–HCl buffer, 50 mM (pH 7.6); [γ-^{32}P]ATP, 1 μM, 0.5 μCi. The reaction tubes were placed on ice and preincubated at 33°C for 6 min (unless otherwise stated). The reaction was initiated by the addition of labeled ATP and incubation was continued for 4 min at 33°C. The reaction was stopped by the addition of 3-fold con-

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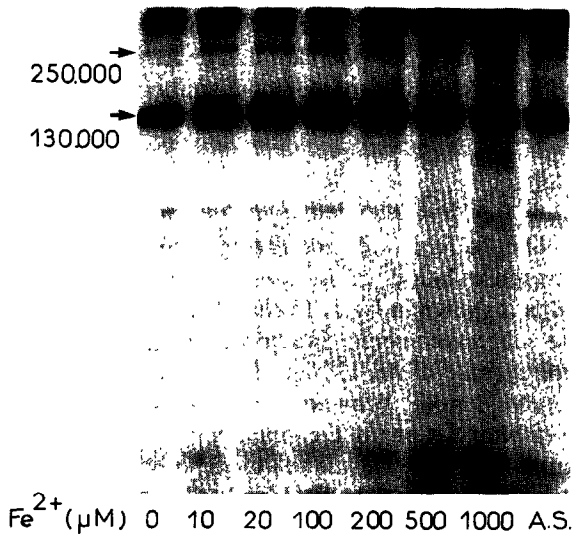

Fig.1. Effect of different concentrations of iron on phosphorylation of mouse liver membrane proteins. Membranes were incubated at 33°C as in section 2 in presence of increasing [Fe^{2+}] (ferrous ammonium sulfate). A control was done with ammonium sulfate alone (200 μM) (A.S.). Electrophoresis and autoradiography were performed as in section 2. The gel was 15% acrylamide.

centrated sample buffer [8] and the membrane components were subjected to electrophoresis in 7.5% or 15% acrylamide 0.1% bisacrylamide slab gels using the discontinuous system [7]. Dissolved samples were allowed to stand at room temperature for 45 min prior to electrophoresis. M_r-standards used for calibration of the gels were: galactosidase, 130000; phosphorylase *a*, 94000; bovine serum albumin, 68000; ovalbumin, 43000; carbonic anhydrase, 30000; soybean trypsin inhibitor, 20100.

After electrophoresis (4 h at 25 mA) with constant current the gels were stained with 0.25%

Table 1

Stimulation of incorporation of ^{32}P by Fe^{2+}

Expt	$-Fe^{2+}$	$+Fe^{2+}$	Stimulation
1	1269	3412	2.70
2	912	5169	5.70
3	970	2989	3.1

Membranes were incubated as usual, the M_r 130000 band was cut and counted by Cerenkov effect

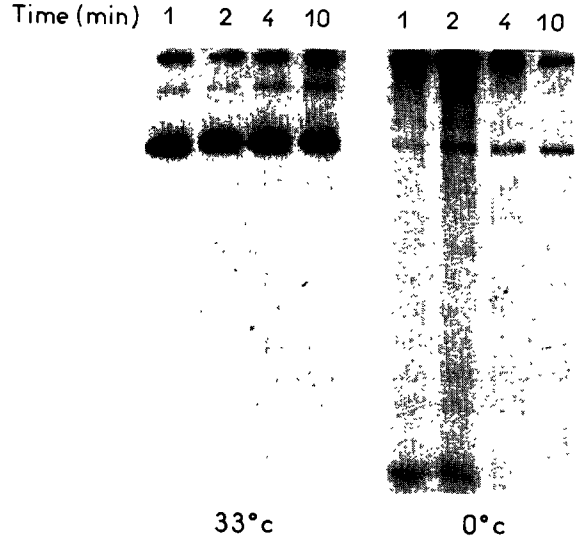

Fig.2. Kinetics of phosphorylation of the M_r 130000 band at 33°C and 0°C. Membranes were incubated in presence of 100 μM Fe^{2+} either at 33°C or at 0°C for different times. The gel was 15% acrylamide.

Fig.3. Compared effects of GTP and ATP. Incubation was effected with: (a,b) [γ-^{32}P]GTP (1 μM, 0.5 μCi); or (c,d) [γ-^{32}P]ATP (1 μM, 0.5 μCi); (a,c) in absence; or (b,d) presence of Fe^{2+}. The gel was 15% acrylamide.

Coomassie brilliant blue R 250. Autoradiography was performed by enclosing a flashed Kodak X-OMAT A R film between the gel and a Dupont lighting screen at −70°C [9] for 1–2 days.

2.3. *Identification of phosphoamino acids after acid hydrolysis*

After autoradiography the labeled polypeptide band was cut out [10], and hydrolysed directly in 6 M HCl at 110°C for 2 h. The phosphoamino acids were separated by electrophoresis at pH 3.5 on cellulose thin-layer plate in one dimension and chromatography in the second dimension [11].

2.4. *Reagents*

[γ-^{32}P]ATP and [γ-^{32}P]GTP were purchased from Amersham (Bucks). Chemicals were from Sigma and from Merck (analytical grade). Iron was in the form of ferrous ammonium sulfate (Merck) (unless otherwise stated).

3. RESULTS

The effect of increasing [Fe^{2+}] on the phosphorylation of mouse liver membrane proteins is shown in fig.1. In the absence of any metal we observed some incorporation of ^{32}P in a polypeptide of M_r 130000. Increasing [Fe^{2+}] from 10 μM–1 mM provokes an increase of the phosphorylation of the polypeptide, with a maximum at 200 μM. The incorporation of ^{32}P decreased at 500 μM Fe^{2+} and $\geq$1 mM Fe^{2+} there

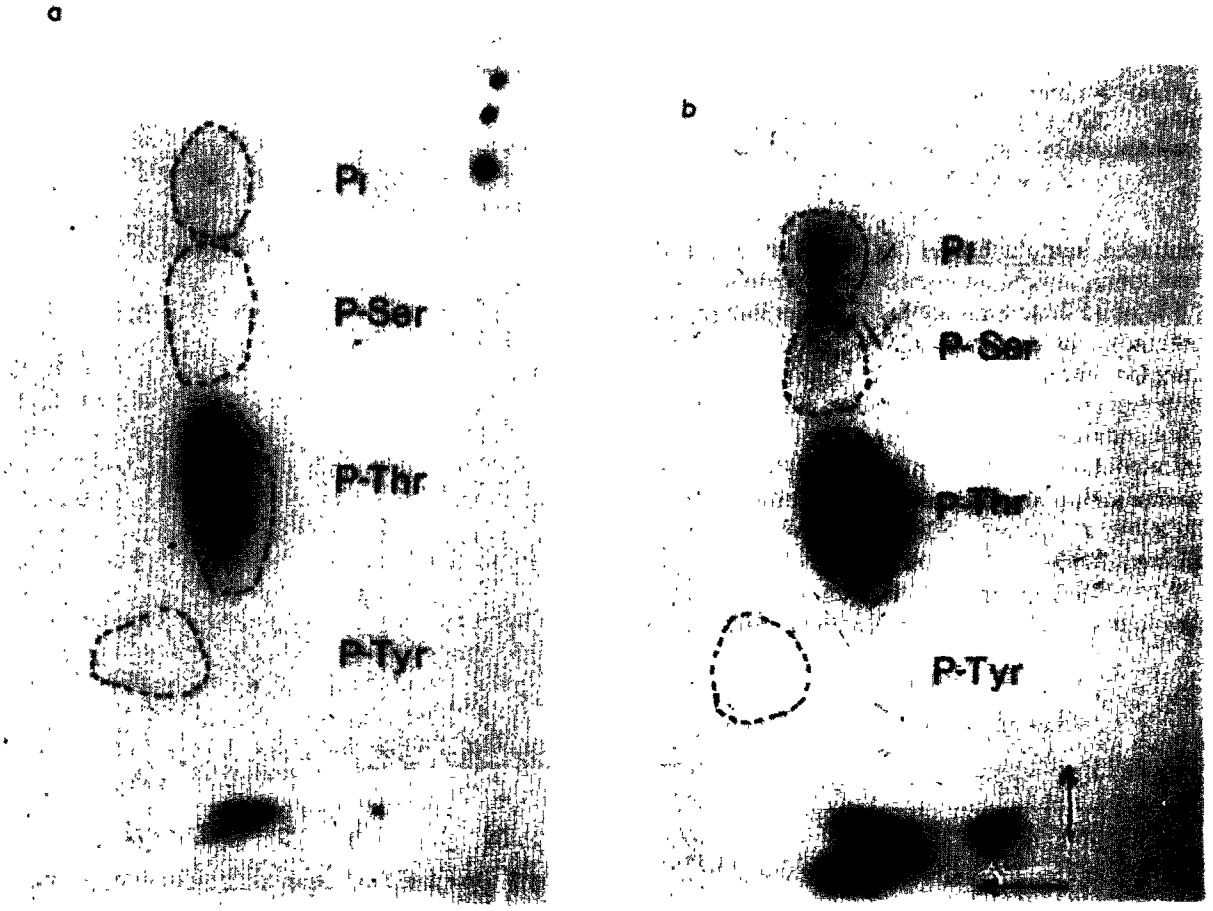

Fig.4. Amino acid determination. Analysis of phosphoamino acids of M_r 130000 band was performed as in section 2: (a) no divalent cation added; (b) with fe^{2+} (100 μM).

was precipitation of material. The Fe^{2+} was added in the form of $(NH_4)_2Fe(SO_4)_2$ which is more stable under experiment conditions. A control was performed in the presence of ammonium sulfate (200 μM) as shown in fig.1 (last lane). There was no enhancement in phosphorylation when compared to the control without Fe^{2+} (fig.1, first lane). $FeCl_2$ and $FeCl_3$ were as effective in the stimulation of phosphorylation as $(NH_4)_2Fe(SO_4)_2$ (not shown). The stimulation by Fe^{2+} varies from 3–6-times according to the experiments (table 1).

It should be noted that along with the M_r 130000 band one observes also the phosphorylation (and stimulation by iron) of a faint band of M_r ~250000.

The kinetics of the reaction in the presence of 100 μM Fe^{2+} at 0°C and 33°C is shown in fig.2. At 33°C there is already a strong incorporation in the M_r 250000 and 130000 polypeptides after 1 min with a maximum at 4 min and a decrease at 10 min. At 0°C the incorporation is minimal. If the membranes are pre-incubated for 10 min at 60°C no phosphorylation is observed in the absence or presence of Fe^{2+} (not shown). Some protein kinases may utilize GTP as phosphate donor as well as ATP. This is not the case here (fig.3) since no incorporation is observed with GTP.

The phosphorylated amino acid on the acid hydrolysate of ^{32}P-labeled M_r-130000 polypeptide cut from the gel was analysed. Fig.4a shows an experiment without cation added and fig.4b the same experiment after addition of 100 μM Fe^{2+}.

In every case, threonine is the only amino acid phosphorylated and its phosphorylation is strongly stimulated by iron. We have indicated the simultaneous presence of the phosphorylated polypeptide of M_r 130000 and of a faint phosphorylated band close to M_r 250000. If the mercaptoethanol was omitted from the sample buffer prior to electrophoresis all of the radioactivity was found in the M_r 250000 band (fig.5a,b). Conversely if 1% mercaptoethanol was added most of the radioactivity was in the M_r 130000 band (fig.5c,d). Thus the M_r 250000 band is probably a dimer.

Other divalent metals, Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Ca^{2+} and Zn^{2+}, at μM as well as mM levels, did not stimulate the phosphorylation of M_r 130000 polypeptide. Only Cu^{2+} gave some stimulation but

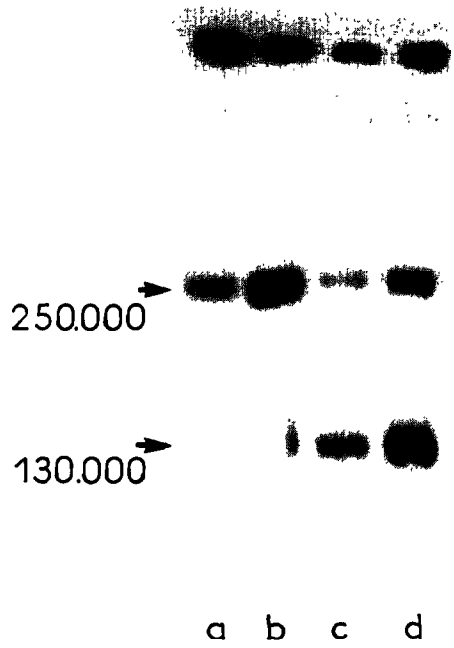

Fig.5. Effect of omission of mercaptoethanol from the sample buffer. Membranes were incubated as usual: (a,b) no mercaptoethanol; (a) without divalent cation; (b) with 100 μM Fe^{2+}; (c,d) mercaptoethanol as usual (1% final) in sample buffer; (c) without divalent cation; (d) with 100 μM Fe^{2+}. The gel was 7.5% acrylamide.

to a lower degree than Fe^{2+}. Transferrin with or without Fe^{2+} had no effect (not shown).

4. DISCUSSION

Our results show that a membrane protein kinase can work in vitro in presence of μM levels of Fe^{2+} in the place of the usual catalysts for protein kinases Mg^{2+} or Mn^{2+} or eventually Ca^{2+}. Fe^{2+} is effective at 20 μM with a maximum at 100–200 μM. Fe^{3+} is also effective.

A membrane polypeptide of M_r 130000 under reducing conditions and M_r 250000 in absence of reducing agents is phosphorylated. The weak but constant phosphorylation observed in absence of any added divalent cation to the medium has the same characteristics as the phosphorylation in presence of Fe^{2+}: phosphorylation of the same polypeptides, M_r 130000 or its dimer and incorporation of phosphate into threonine residues. It is probably the same kinase activated by the low amounts of Fe^{2+} present in the solutions (although

bidistilled water and purest reagents when possible have been used). Fe^{2+} may eventually be present in membranes either naturally or picked up during the preparation.

In microsomes of rat liver, a system of phosphorylation has been described [12] which has some characteristics similar to that described here. A crude fraction from microsomes may be phosphorylated in vitro without addition of divalent cations [12]. The phosphorylation of two polypeptides of M_r 145000 and 130000 was observed and also the presence of dimers of M_r ~275000 if the mercaptoethanol is omitted from the SDS gel. However, they did not observe the stimulation of phosphorylation by Fe^{2+} as they used much higher concentrations of divalent cations (at 20 mM), which in our experiments are inhibitory.

We have not found phosphorylation of the M_r 130000 polypeptide in purified mouse liver microsomes free of plasma membranes and it is probably in the membranes contaminating the microsomes that the reaction described in [12] takes place. The presence of two phosphorylated proteins may be caused by a proteolytic degradation. With membranes of rat liver, we have also found two phosphorylated polypeptides but of M_r ~130000 and 120000, whereas in mouse only one phosphorylated polypeptide of M_r 130000 was found.

At present we do not know, as for some protein kinases such as SRC kinase [13] or EGF receptor [14], if it is an autophosphorylation of the kinase or if the kinase and the substrate are different.

Stimulation of cell growth by iron salts or peptide containing iron has been observed not only in bacteria but also in mammalian cells. Growth modulating peptides containing Fe^{2+} or Cu^{2+} have been described [15,16]. We have found that transferrin has no effect on the phosphorylation of membrane proteins. The polypeptide of M_r 130000 phosphorylated in presence of Fe^{2+} has an M_r clearly distinct from the transferrin receptor which is 90000 and may be phosphorylated on serine residues [17].

Further experiments are necessary to see if this polypeptide is phosphorylated after addition of iron to the medium of cultured cells and to identify the phosphorylated protein and its function.

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